Effects of Temperature on the Coupled Activities of the Vanadate-Sensitive Proton Pump from Maize Root Microsomes

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ABSTRACT

The mechanism by which proton transport is coupled to ATP hydrolysis by vanadate-sensitive pumps is poorly understood. The effects of temperature on the activities of the vanadate-sensitive ATPase from maize (*Zea mays*) roots were assessed to provide insight into the coupling mechanism. The initial rate of proton transport had a bell-shaped dependence on temperature with an optimal range between 20 and 30°C. However, the rate of vanadate-sensitive ATP hydrolysis increased as the temperature was raised from 4 to 43°C. The differential sensitivity of proton transport to temperatures above 30°C was also observed when the ATPase was reconstituted into dioleoylphosphatidylcholine vesicles. Inhibition of proton transport with temperatures above 30°C was associated with higher rates of proton leakage from the membranes. In addition, proton transport was more inhibited than ATP hydrolysis at temperatures below 10°C. Reduced rates of proton transport at lower temperatures were not associated with higher rate of proton conductivity across the membranes. Therefore, the preferential inhibition of proton transport at temperatures below 10°C may reflect an effect of temperature on the coupling between proton transport and ATP hydrolysis within the vanadate-sensitive ATPase.

Plant cells contain two predominant classes of proton translocating ATPase which energize solute transport (5, 16). Both of these proton pumps convert the chemical energy from the hydrolysis of ATP to ADP into an electrochemical gradient by the unidirectional movement of protons across a membrane. The two classes of proton pumps are commonly referred to as the vacuolar- and plasma membrane-type ATPase based on their localization in the tonoplast and plasma membranes, respectively (14, 16). Biochemically, the two classes of ATPase can be distinguished by their differential sensitivity to nitrate and vanadate, formation of a covalent phosphorylated intermediate, and polypeptide composition (14).

With vacuolar-type ATPase from maize (*Zea mays*) root tonoplast vesicles, the temperature dependence of the proton transport differed from that of ATP hydrolysis (17). The initial rate of proton transport declined as the temperature was increased above 25°C, whereas the rate of ATP hydrolysis by the pump continued to increase. These results supported the hypothesis that proton transport and ATP hydrolysis by the vacuolar ATPase were indirectly coupled. In this paper, the nature of the coupling mechanism of the plasma membrane-type ATPase from maize root has been investigated in a similar manner by examining the temperature dependence of proton transport and ATP hydrolysis.

MATERIALS AND METHODS

Preparation of Membrane Vesicles

Maize seedlings (*Zea mays* L., cv WF9 × Mo17) were grown on filter paper moistened with $CaCl_2$ (13). KI-washed microsomes were prepared from 3-d-old roots as described previously (3). The ATPase in these microsomes were reconstituted into di-18:1 PC[1] liposomes by a detergent dilution protocol using deoxycholate as described previously (3), using a lipid to protein ratio of 20 to 1 by weight. Protein concentration was determined by a modification of the Lowry method after precipitation by TCA in the presence of deoxycholate (2).

Proton Transport and ATP Hydrolysis Assays

Proton transport was followed primarily by changes in the absorbance of AO at 492.5 nm as described by de Michelis *et al.* (5). Assays were conducted with Beckman DU-70[2] spectrophotometer equipped with a thermoregulated cell holder attached to a circulating water bath. Temperature in a cuvette containing assay medium adjacent to the sample was monitored. Typically, 100 to 200 μL of membrane vesicles were diluted to 2 mL with assay medium containing 17.5 mM Mes titrated to pH 6.45 with bis-tris-propane, 50 mM KNO_3, 1 mM EGTA, 2.5 mM $MgSO_4$, 25 mM glucose, and 7.5 μM AO. After equilibration at the desired temperature for 6 min, proton transport was initiated by the addition of 20 μL of 0.2 M ATP titrated to pH 6.45 with bis-tris-propane.

The development of the pH gradient generated by the vanadate-sensitive proton pump was analyzed according to a kinetic model described in detail elsewhere (4). According to

[1] Abbreviations: di-18:1 PC, dioleoylphosphatidylcholine; A_s, change in absorbance at steady-state; AO, acridine orange; k_L, rate constant for dissipation of proton gradient after ATP hydrolysis by the pump has been inhibited; k_p, rate constant describing those processes hindering the development of a proton gradient; R_{ATP}, rate of ATP hydrolysis; R_H, initial rate of proton transport.

[2] Reference to brand and firm does not constitute endorsement by U.S. Department of Agriculture over others of similar nature.

the model, net proton transport ($d\delta/dt$) at a given time, t, may be represented by:

$$(d\delta/dt) = m \cdot R_{ATP} - k_p \cdot \delta \quad (1)$$

where R_{ATP} is the rate of ATP hydrolysis by the pump, m is the stoichiometry of proton transport to ATP hydrolysis, δ is the net amount of transported protons, and k_p is the rate constant for those processes hindering the development of a proton gradient. The rate constant, k_p, considers such processes as back pressure inhibition, pump slippage, and proton leakage. Proton transport by the vanadate-sensitive proton pump from maize roots has been shown to conform to the above relationship (4). Adherence to this model has two implications: the stoichiometry of proton transport to ATP hydrolysis catalyzed by the pump is constant when the rate of ATP hydrolysis is also constant under a particular set of experimental conditions; and a steady-state gradient is achieved when the magnitude of proton transport by the pump, *i.e.* $m \cdot R_{ATP}$, is equal to the magnitude of the inhibitory processes, $k_p \cdot \delta$. In addition, the time course for the generation of the proton gradient can be reproduced by knowing two of the following three parameters: R_H, A_s, and k_p.

Direct experimental evidence demonstrated that changes in AO absorbance can be related directly to net proton transport (4). Therefore, k_p can be estimated from the relationship $In\ (1\text{-}A_t/A_s) = k_p \cdot t$, where A_t is the change in absorbance at time t, A_s is the steady-state change in AO absorbance, and t is time (4). Also, the initial rate of proton transport, R_H, can be determined conveniently by: $R_H = k_p \cdot A_s$ (4). Typically, duplicate assays were performed and each experiment was conducted at least twice. Standard errors of the means are reported where the variation exceeds the size of the data symbol.

In those experiments in which the collapse of the steady-state proton gradient was followed, a steady-state proton gradient was established, and then 100 units of hexokinase were added to remove the remaining ATP rapidly (4). The collapse of the pH gradient followed first-order kinetics (4), so that the rate constant, k_L, was estimated by:

$$In\ (A_t/A_s) = k_L \cdot t \quad (2)$$

Normally, after the establishment of a steady-state pH gradient (5–10 min after the addition of ATP), 100 μL aliquots of assay medium were removed and inorganic phosphate release was determined by the formation of the malachite green-ammonium molybdate complex (18). Parallel assays conducted in the presence of 0.2 mM vanadate were performed and the difference was attributed to the vanadate-sensitive, nitrate-insensitive proton pump. The rate of ATP hydrolysis was constant throughout the development of the pH gradient (4). Typically, assays were conducted with three replications. Standard errors of the means are reported where the variation exceeds the size of the data symbol.

Results with AO were confirmed by measuring proton transport by changes in the fluorescence of either 9-amino-6-chloro-2-methoxyacridine or quinacrine. The AO in the assay medium was substituted with 10 μM of either of the two dyes. Fluorescence was monitored using excitation and emission wavelengths of 425 and 508 nm, respectively, for quinacrine and 409 and 474 nm, respectively, for 9-amino-6-chloro-2-methoxyacridine.

RESULTS

Effect of Temperature on the Coupling of the Vanadate-Sensitive Proton Pump

Effects of temperature on the development of the proton gradient generated by the vanadate-sensitive ATPase from maize root KI-washed microsomes were investigated using changes in the absorbance of AO. As the temperature was increased from 7 to 20°C, the R_H increased progressively (Fig. 1). Between 20 and 30°C, there was little change in R_H. Between 30 and 39°C, proton transport decreased. Above 39°C, no pH gradient was detected. Thus, R_H showed a bell-shaped dependence on temperature with an optimal range between 20 and 30°C. The decrease in R_H as temperature was either increased or decreased from the optimal range of 20 to 30°C corresponded to a change in the time course of the development of the pH gradient as reflected by an increase in k_p, a measure of the processes inhibiting net transport (4).

Decreases in proton transport at higher temperature were not associated with an inhibition of ATP hydrolysis (Fig. 1). Rates of ATP hydrolysis increased throughout the temperature range of 5 to 45°C. Thus, hydrolysis of ATP and proton transport by the vanadate-sensitive pump responded to temperature differently. A similar differential response between proton transport and ATP hydrolysis to temperature was observed with the nitrate-sensitive proton pump from maize root tonoplast vesicles (17).

The temperature dependence of the vanadate-sensitive proton pump reconstituted into di-18:1 PC vesicles was similar

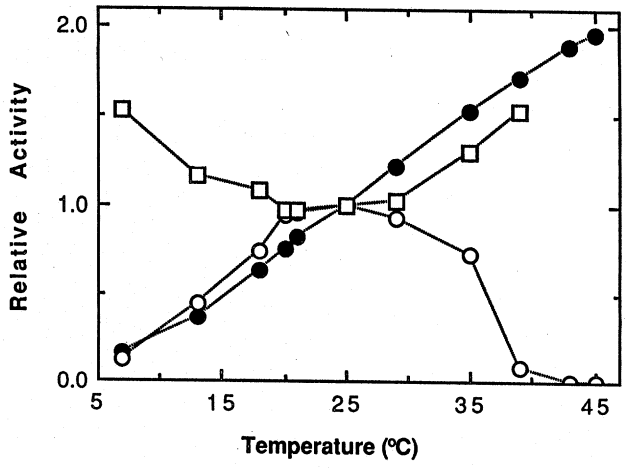

Figure 1. Effects of temperature on vanadate-sensitive ATP hydrolysis, the R_H, and k_p with KI-washed microsomes. Proton transport was assessed at various temperature between 7 and 45°C. The data were analyzed according to the kinetic model as described previously (4) to determine R_H (○) and k_p (□), and plotted relative to the values obtained at 25°C, 0.12 ± 0.01 $\Delta A\ min^{-1}$ mg $protein^{-1}$, and 0.42 ± 0.01 min^{-1}, respectively. Samples were also assayed for vanadate-sensitive ATP hydrolysis (●), and the data are plotted relative to the value obtained at 25°C, 163 ± 13 nmol Pi min^{-1} mg $protein^{-1}$.

to that of native microsomes (Fig. 2A). Values for R_H had a pronounced optimum between 20 and 30°C, whereas rates of ATP hydrolysis increased with temperature up to 40°C. The apparent bell-shaped temperature dependence of proton transport was not unique to the AO assay. When proton transport was assayed by changes in the fluorescence of either quinacrine or 9-amino-6-chloro-2-methoxyacridine, maximum values for R_H were found between 20 and 30°C (data not shown). Addition of valinomycin had no effect on proton transport even at temperatures below 20°C (data not shown).

As reported in Figure 1 with native vesicles, the value for k_p varied with temperature with reconstituted vesicles (Fig. 2B). Maximum values for k_p were found at temperatures below 10°C and above 30°C. Minimal values of k_p were associated with maximum rates of R_H. Therefore, lower rates of proton transport were associated with greater contribution of those processes hindering the formation of a pH gradient such as proton leakage and pump slippage.

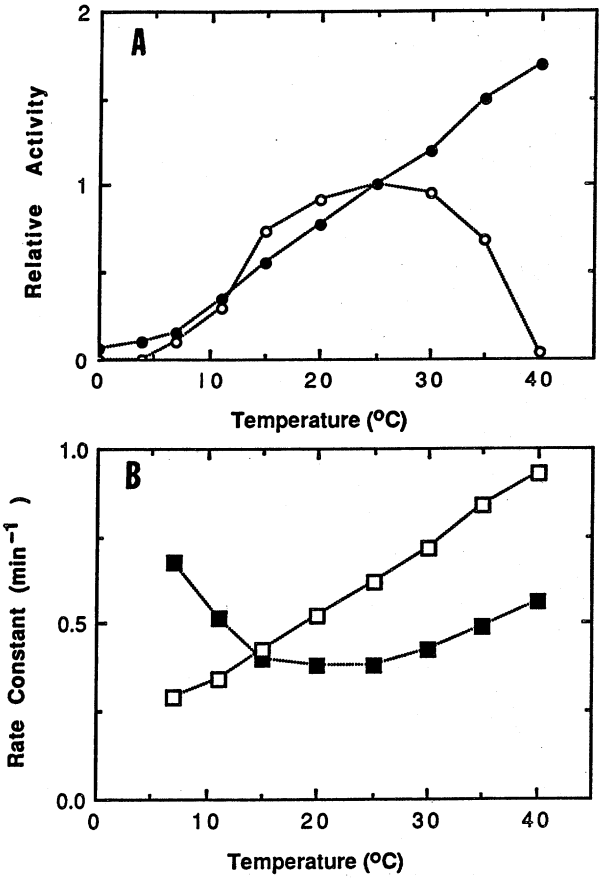

Figure 2. Effects of temperature on the activity of reconstituted vanadate-sensitive ATPase and membrane leakage. ATPase in KI-washed microsomes were reconstituted into di-18:1 PC vesicles as described previously (3) and then assayed for vanadate-sensitive ATP hydrolysis (●) and proton transport (○) in panel A. Data are plotted relative to the values of obtained at 25°C, which averaged 0.12 ± 0.01 $\Delta A\ min^{-1}$ mg $protein^{-1}$ for R_H and 158 ± 10 nmol Pi min^{-1} mg $protein^{-1}$ for ATP hydrolysis. Proton transport by reconstituted vesicles was analyzed by the kinetic model as previously described (4) to estimate k_p (panel B, Δ). After the proton pump had established a steady-state pH gradient, the ATPase was inhibited rapidly by addition of hexokinase (5). The rate of collapse of the gradient was analyzed by first-order kinetics to determine k_L (panel B, ▲).

The effect of temperature on k_L, rate constant for the dissipation of a steady-state pH gradient after the proton pump is inhibited, was determined to ascertain the changes in the conductance of the membrane to protons (Fig. 2B). The absolute value for k_L at 25°C was greater than that of k_p, at temperatures above 11°C in agreement with an earlier finding by this laboratory (4). At temperatures below 15°C, k_p was greater than k_L. The values for k_L increased almost threefold as the temperature was raised from 4 to 42°C. The increase in k_L above 25°C paralleled increases in k_p. Between 7 and 25°C, k_L increased gradually. This change is in sharp contrast to that observed with k_p. As the temperature was increased from 7°C, k_p decreased toward a minimum at 25°C.

The validity of the AO assay for measuring proton transport at temperatures above 30°C was examined further. KI-washed microsomes reconstituted with di-18:1 PC were prepared and the response of AO to an imposed pH gradient of 1.2 units was determined in "pH jump" experiments. The change in AO absorbance to an imposed pH gradient was relatively constant between 10 and 40°C, averaging 0.30 ± 0.07 A mg $protein^{-1}$ (data not shown). The rate constants for the dissipation of an imposed pH gradient were very similar to those obtained for k_L, averaging 0.6 min^{-1} at 25°C (data not shown). The effects of temperature on the rate of dissipation parallelled the effects observed with k_L (data not shown).

DISCUSSION

The temperature optimum for proton transport by the vanadate-sensitive proton pump from maize roots was found to be between 20 and 30°C with both native and reconstituted vesicles (Figs. 1 and 2). Such results are in contrast to that reported previously for the plasma membrane-type ATPase from barley roots, which had greater activity at 40°C than at 30°C (7). The temperature optimum for ATP hydrolysis for maize root vanadate-sensitive proton pump was found above 40°C (Figs. 1 and 2), which is similar to that reported previously (6, 7, 10, 15, 20). Therefore, proton transport and ATP hydrolysis by the maize root plasma membrane-type proton pump had different temperature optima. The different temperature optima for ATP hydrolysis and proton transport, and the bell shaped temperature dependence for proton transport for the vanadate-sensitive ATPase is similar to that found previously for the maize root tonoplast ATPase (17). The effects of temperature on the tonoplast-type ATPase were interpreted in terms of an indirect coupling mechanism between transport and ATP hydrolysis (17).

Maximum values for the initial rate of proton transport, R_H, were associated with the minimal values for k_p, the rate constant quantifying the magnitude of the processes that decrease net transport (Figs. 1 and 2). Therefore, the decrease of proton transport at temperatures either above or below the optimum were associated with an increase in processes, such as proton leakage and pump slippage. Temperature effected proton leakage through the membrane in the absence of an active proton pump. The rate of collapse of the proton gradient, k_L, increased as temperature increased above 25°C in a

manner roughly parallel to changes in k_p (Fig. 2). The obvious explanation for the decreases in proton transport at temperatures above 25°C is that increasing the temperature increased the leak. The reason for the enhanced proton leakage is not known but could arise from increases in membrane fluidity, proton diffusion, or lipolytic degradation. Enhanced proteolysis probably does not contribute to the increased leakage because these membranes exhibit little if any peptide degradation (3).

At temperatures below the optimum of 20 to 30°C, the response of k_p and k_L were divergent (Fig. 2). Values for proton leakage declined as temperature was decreased from 25 to 7°C, whereas k_p increased. This differential response of k_p and k_L to temperature indicates that there was an ATP-dependent process that increased with decreasing temperature and was associated with decreased proton transport.

One interpretation of differential responses of proton transport and k_p from that of ATP hydrolysis and k_L at temperatures below 20°C may be an indirect coupling mechanism between proton transport and ATP hydrolysis by the pump. An indirect mechanism implies that ATP hydrolysis and proton transport do not share an intermediate step (8). Instead, there is a distinct coupling mechanism linking the two processes. Under such a model, decreasing the temperature from 25 to 7°C could reduce the rate of either coupling or proton transport more than ATP hydrolysis, leading to an increase in k_p. Recently, Inesi and de Meis (9) have proposed that the Ca transport and ATP hydrolysis by the sarcoplasmic reticulum pump are indirectly coupled. An indirect coupling mechanism has been proposed for the maize root tonoplast H^+-ATPase to explain the increased sensitivity of proton transport to nitrate (18), divalent cations (19), and temperature (17) relative to ATP hydrolysis. Therefore, the reaction mechanism of the plasma membrane-type ATPases may be modeled better by an indirect coupling mechanism rather than a direct mechanism, in which transport and ATP hydrolysis share at least one step (12) as exemplified by the Post-Alber cycle of the Na, K, ATPase (11) and the coupling between nucleotide displacement and reorientation of the Ca binding sites with the sacroplasmic reticulum ATPase (1).

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